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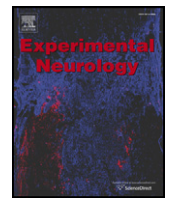
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Research Paper

NOX4 is an early initiator of neuropathic pain

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ABSTRACT

Treatment of neuropathic pain remains challenging as the etiology is heterogeneous and pathomechanisms are incompletely understood. One possible mechanism is oxidative stress due to unphysiological reactive oxygen species (ROS) formation. The only known dedicated enzymatic source of ROS are NADPH oxidases of which the type 4 isoform (NOX4) has been suggested to be involved in the subacute and chronic phase of neuropathic pain. Here, we aim to translate this finding into a treatment strategy by examining the efficacy of the NOX1/4-specific inhibitor GKT136901 using the chronic constriction injury (CCI) mouse model of neuropathic pain. Unexpectedly, post-nerve lesion treatment using GKT136901 was ineffective to reduce pain-related behavior after CCI. We therefore re-investigated the role of NOX4 using an independent KO mouse model. Early after CCI we found an increase in pro-inflammatory cytokines, ROS formation and the oxidative stress marker nitrotyrosine in the lesioned nerve together with an upregulated *Nox4* gene expression. In NOX4 KO mice, mechanical allodynia was markedly reduced from day 4 after nerve injury as were all ROS related and acute biomarkers. In addition, we observed a reduction in the CCI-induced upregulation of pro-inflammatory cytokines in the sciatic nerve and dorsal root ganglia along with NOX4-deficiency. Thus, we conclude that NOX4 is involved in the development of neuropathic pain states by producing oxidative stress and subsequent cytokine dysregulation at the lesion site. This appears at very early stages immediately after nerve injury explaining ineffectiveness of post-acute pharmacological NOX inhibition. We suggest that future target validation of NOX4 should now focus on defining the possible therapeutic window in human neuropathic pain.

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1. Introduction

Neuropathic pain is a major public health problem that affects approximately 6% of the adult population worldwide. Despite several therapeutic options, treatment of neuropathic pain remains challenging because of the heterogeneous etiology and the multifactorial mechanisms underlying this disease (Finnerup et al., 2015). Nerve damage induces a complex machinery of pathophysiologic events in the periphery including neuronal hyperexcitability, changes in perineuronal homeostasis, altered gene expression, and the induction of a strong immune response (Rotshenker, 2011). These mechanisms together with secondary events in the central pain processing pathways contribute to the development and maintenance of a steady-state pain condition.

Several studies have indicated that oxidative stress induced by an imbalance in redox homeostasis is critically involved in the induction

of neuropathic pain (Jaggi and Singh, 2011; Kallenborn-Gerhardt et al., 2013; Salvemini et al., 2011). Oxidative stress occurs due to overproduction of free radicals, i.e. reactive oxygen species (ROS) such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2) together with a failure of antioxidant defense mechanisms (Altenhöfer et al., 2012). Intracellular accumulation of ROS and disturbances in the cellular redox state are the consequences that then induce not only to general oxidative stress responses like DNA fragmentation, breakdown of signaling pathways, and tissue damage, but also neuronal degeneration and sensitization in pain conditions (Salvemini et al., 2011; Schafer and Buettner, 2001). Accordingly, therapeutically balancing of ROS by the administration of antioxidants or free radical scavengers appears encouraging, but to date, efforts to inhibit ROS have not yielded beneficial effects in pain conditions (Kallenborn-Gerhardt et al., 2013; Schwartz et al., 2008; Steinhubl, 2008; Yowtak et al., 2013). Prevention of ROS generation, rather than scavenging already formed free radicals, represents another possibility to target oxidative stress (Altenhofer et al., 2014; Padi and Kulkarni, 2008).

One source of ROS production is the enzyme family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX). Compared

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with other ROS-producing enzymes NOX stand out as ROS generation is their primary function (Altenhofer et al., 2014; Opitz et al., 2007). The rodent genome encodes four genes that contain the catalytic NOX subunit, namely *Nox1*, *Nox2*, *Nox3*, and *Nox4* (Altenhöfer et al., 2012). This electron-transferring subunit is constitutively inactive in resting cells and generates ROS only upon activation, e.g. after noxious stimuli (Salvemini et al., 2011). While NOX2 activation is predominantly associated with innate immunity mediated host defense (Lam et al., 2010) and NOX1 with blood pressure control and related vascular mechanisms (Gavazzi et al., 2006), NOX4 was shown highly expressed under ischemic conditions in the central nervous system, e.g. ischemic stroke (Kleinschnitz et al., 2010; Suzuki et al., 2012).

Recent evidence identified activation of NOX as a causative factor that contributes to inflammatory or neuropathic pain role in the peripheral nervous system (Ibi et al., 2008; Kallenborn-Gerhardt et al., 2012, 2014; Kim et al., 2010). Interestingly, NOX4 was reported to be important in the subacute and late-phase of neuropathic pain but to be dispensable for basal sensitivity and for inflammatory pain conditions as evaluated in a NOX4 KO mouse model (Kallenborn-Gerhardt et al., 2012). Injury-induced ROS production as well as subsequent dysmyelination processes of peripheral nerves were identified as the potential underlying mechanisms. Tamoxifen-induced *Nox4* gene deletion was effective in reducing pain behavior in late stages of neuropathic pain. Here, we first aimed to evaluate the effect of a pharmacological NOX4 blockade using the NOX1/4 inhibitor GKT136901 in the chronic constriction injury (CCI) mouse model of neuropathic pain. Since post-injury application of GKT136901 was ineffective, we re-investigated the role of NOX4 on neuropathic pain behavior, oxidative stress, and inflammatory processes in a different NOX4 KO model and found NOX4 signaling important for the development of neuropathic pain very early after nerve injury.

2. Material and methods

2.1. Animals

All animal experiments were approved by local state authorities (Regierung von Unterfranken), comply with the ARRIVE guidelines and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments as well as the German Animal Welfare Act (German Ministry of Agriculture, Health and Economic Cooperation). Experiments were performed on adult male mice at 8–15 weeks of age weighing 25–35 g. The generation of NOX4-deficient (*Nox4*^{−/−}) mice has been described previously (Kleinschnitz et al., 2010). Wild-type (WT) littermates (*Nox4*^{+/+} mice) served as controls. Pharmacological inhibition of dual NOX1/4 by the NOX4 inhibitor GKT136901 (Vendrov et al., 2010) was performed in 8-week old male C57BL/6 mice (Charles River Laboratories, Research Models and Services GmbH, Sulzfeld, Germany). Mice were group-housed in boxes in a temperature- and humidity-controlled environment with a 12/12 h light-dark cycle with standard rodent chow and water ad libitum. The minimal number of animals needed to obtain statistical significance was used in the present study to reduce animal numbers and to minimize animal distress. In total, 61 *Nox4*^{−/−}, 64 *Nox4*^{+/+} and 15 C57BL/6 mice were included in the study. For experimental group planning we extrapolated pain levels from large numbers of own previous experiments using the CCI pain model, assumed the pain levels of *Nox4*^{−/−} mice to 50%, and determined the significance level to 0.05 and the power to 0.8 (beta = 0.2).

2.2. Chronic constriction injury (CCI)

CCI was performed according to the original description in rats (Bennett and Xie, 1988) modified for mice as reported previously (Schäfers et al., 2003). Surgical procedures were carried out under barbiturate anesthesia using sodium pentobarbital (Merial GmbH,

Hallbergmoos, Germany) at 50 mg/kg i.p. Briefly, both sciatic nerves were exposed and three ligatures (7–0 prolene) were placed around the right sciatic nerve with 1-mm spacing. Ligatures were tightened until they elicited a brief twitch in the respective hind limb. For sham surgery, sciatic nerves were exposed in the same way but without placing ligatures. Surgeries were performed while being blinded to the experimental groups.

2.3. Behavioral testing

Behavioral tests were performed on Day −3 and on Day −1 before CCI to obtain baseline values, and on Days 4, 8, 14, 21, 28, and 35 after CCI. All behavioral testing was done by the same investigator unaware of the treatment and the respective genotypes. For all tests, mice were adapted to the testing environment for at least 1 h before testing.

a) Assessment of tactile allodynia (von Frey hair-induced paw-withdrawal threshold)

Mice were individually placed on an elevated metallic wire mesh floor in polyethylene cages. The von Frey filaments (Stoelting Co., Wood Dale, USA) were inserted through the mesh floor and applied to the plantar surface of the hind paw. The force required to bend the hairs ranged from 0.004 to 28.84 g. Hairs were applied six times each on the basis of the up-and-down method as described previously (Chaplan et al., 1994; Geis et al., 2010). The time interval between two trials was at least 1 min on the same paw and at least 30 s on the alternate paw. A brisk withdrawal of the paw was considered a positive response. The 50% withdrawal threshold (i.e. force of the von Frey hair to which an animal reacts in 50% of the presentations) was recorded.

b) Assessment to thermal hyperalgesia (withdrawal latency to radiant heat)

The withdrawal latency to heat stimulation was tested using a Plantar Test Instrument (Ugo Basile, Comerio, Italy). The time until the animals reacted with withdrawal to the stimulation by a radiant heat source was determined automatically. Each testing period consisted of a minimum of three presentations for each hind paw. Means of three tests were calculated and taken for further analysis (Geis et al., 2011). The time interval between two trials was at least 1 min. All animals reacted within the 20 s timeframe of the maximal stimulation period.

2.4. Pharmacological NOX inhibition and drug delivery

In a therapeutic approach, we used the small molecule dual NOX1/4 inhibitor GKT136901 (2-(2-chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo[4.3-c]pyridine-3,6(2H,5H)-dione), which was dissolved in 0.5% carboxymethyl cellulose, 0.25% polysorbate 20, and 1% dimethyl sulfoxide according to previously published protocols (Vendrov et al., 2010). GKT136901 has been shown to reduce ROS generation in vivo when delivered orally in mice (Gaggini et al., 2011; Teixeira et al., 2016; Vendrov et al., 2010). We therefore administered GKT136901 (10 mg/kg once daily) through oral gavage using 20-gauge gavage needles beginning from Day 1 after CCI to Day 21. We used C57BL/6 mice, which were randomly selected to receive either GKT136901 or vehicle (i.e. buffer solution without GKT136901). The experimenter treating the animals was blinded to treatment allocation.

2.5. Euthanasia and tissue processing

After the final testing or at the indicated time points of tissue analysis, mice were deeply anesthetized using sodium pentobarbital and perfused through the left atrium with 20 ml phosphate-buffered saline (PBS). Bilateral sciatic nerves and L4 and L5 dorsal root ganglia (DRG) as well as lumbar spinal cord were removed. For immunohistochemistry, tissue was then embedded into optimal cutting-temperature compound (TissueTek®, Sakura Finetek Europe B.V., Alphen aan den Rijn,

The Netherlands) and snap-frozen in isobutene placed in liquid nitrogen. For gene expression analysis, native tissue was saved in RNAlater (QIAGEN GmbH, Hilden, Germany) and immediately stored at -80°C for further processing.

2.6. Gene expression analysis

Tissue homogenization, RNA isolation and real-time, quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed as described previously (Göb et al., 2015; Kleinschnitz et al., 2004). Briefly, total RNA from the ipsilateral sciatic nerve, ipsilateral pooled L4/L5 DRG, and lumbar spinal cord was prepared from *Nox4*^{-/-} mice and control littermates (*Nox4*^{+/+}) on Days 1, 4, 8, 21, and 35 after CCI with a Micra D-8 Homogenizer (ART Prozess- & Labortechnik GmbH, Müllheim, Germany) using the TRIzol® reagent (Invitrogen GmbH, Darmstadt, Germany) and was quantified spectrophotometrically. Then, 250 ng of total RNA were reverse transcribed with TaqMan® Reverse Transcription Reagents (Applied Biosystems GmbH, Frankfurt, Germany) according to the manufacturer's protocol using random hexamers.

Relative gene expression levels of murine *NADPH oxidase 2* (*Nox2*) (assay ID: Mm01287743_m1, Applied Biosystems), *NADPH oxidase 4* (*Nox4*) (assay ID: Mm00479246_m1, Applied Biosystems), *interleukin-1 beta* (*Il-1β*) (assay ID: Mm 00434228_m1, Applied Biosystems), *tumor necrosis factor-alpha* (*Tnf-α*) (assay ID: Mm 00443258_m1, Applied Biosystems), and *transforming growth factor-beta1* (*Tgf-β1*) (assay ID: Mm 00441724_m1, Applied Biosystems) were quantified with fluorescence TaqMan® technology. GAPDH (TaqMan® Pre-Developed Assay Reagents for Gene Expression, part number: 4352339E, Applied Biosystems) was used as an endogenous control. The PCR was performed with equal amounts of cDNA and water control in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the TaqMan® Universal PCR Master Mix (2 × concentration) (Applied Biosystems). Each sample was measured in triplicate and data points were examined for integrity by analysis of the amplification plot. The comparative threshold cycle method was used for relative quantification of gene expression as described previously (Livak and Schmittgen, 2001).

2.7. Immunohistochemistry and oxidative chemistry biomarkers

Cryosections (10 μm) of the sciatic nerve from *Nox4*^{-/-} mice and control littermates (*Nox4*^{+/+}) were prepared using a cryostat (Leica Microsystems GmbH, Wetzlar, Germany). For immunohistochemical analysis of reactive nitrogen species (RNS), a rabbit polyclonal antibody to nitrotyrosine (Fisher Scientific GmbH, Schwerte, Germany, EMD Millipore 06284 Cat. 06-284) was applied at a final dilution of 1:100. Sections were incubated at room temperature for 2 h, washed three times for 10 min in 1 × PBS and incubated at room temperature for 45 min with Cy2-conjugated goat anti-rabbit secondary antibody (1:300, Dianova GmbH, Hamburg, Germany) (Kleinschnitz et al., 2010). For quantification of nitrotyrosine-positive cells per mm², identical sections from the sciatic nerve were selected and cell counting was performed from four subsequent slices (40-μm distance between slices). The presence of ROS was visualized on cryosections from the sciatic nerves using dihydroethidium (DHE) (D7008, 2 μM stock, Sigma-Aldrich Chemie GmbH, Munich, Germany Sigma). DHE is a fluorescent dye that after reaction with ROS is converted into the red fluorescent compound ethidium, which intercalates into double-stranded DNA. Oxidative cell damage was calculated by measuring the mean fluorescence intensity of oxidized ethidium per nerve section from four subsequent slices (40-μm distance between slices) of *Nox4*^{-/-} mice or controls (*Nox4*^{+/+}). DNA was counterstained using Hoechst reagent (33,342, Sigma-Aldrich Chemie GmbH). Negative controls included omission of the primary or secondary antibodies and produced no signal. All sections were viewed and images were acquired with a Nikon Eclipse 50i microscope equipped with the DS-U3 DS camera control unit. Images

were analyzed with NIS-Elements software (Nikon Systems, Tokyo, Japan).

2.8. Statistical analysis

For statistical analysis SigmaPlot 12.0 (Systat Software GmbH, Erkrath, Germany) was used. The data were tested for normal distribution using the Shapiro-Wilk test. Behavioral data was analyzed using a 2-way repeated measures ANOVA and a post-hoc Holm-Sidak pairwise multiple comparison procedure. Data from the qRT-PCR were analyzed by 2-way ANOVA with post-hoc Bonferroni adjustment for *P* values. For histological data, an unpaired, 2-tailed Student's *t*-test was applied. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Pharmacological NOX4-blockade by GKT136901 is not effective in reducing CCI-induced mechanical allodynia

NOX4 has been reported to contribute to neuropathic pain behavior in the late phase after peripheral nerve injury (Kallenborn-Gerhardt et al., 2012). We tested here whether pharmacological NOX4 inhibition by therapeutic application of the dual NOX1/4 inhibitor GKT136901 (Teixeira et al., 2016; Vendrov et al., 2010) is effective in the CCI mouse model. GKT136901 was applied from Day1 after CCI in 8-week old male C57BL/6 mice after random allocation to receive either GKT136901 or vehicle. Before CCI, both treatment groups had similar baseline levels of mechanical withdrawal thresholds and heat-induced withdrawal thresholds. Post-injury treatment with GKT136901 did not result in improvement of disease signs (Fig. 1). After CCI GKT136901 treated mice had similar mechanical withdrawal thresholds as control mice throughout the entire experimental period beginning from Day 4 after CCI (*P* > 0.05 for Days 4, 8, 14, and 21 after CCI; Fig. 1A). Withdrawal latencies to radiant heat were also not different between the experimental groups (*P* > 0.05 for Days 4, 8, 14, and 21 after CCI; Fig. 1B).

3.2. Nox4 is upregulated in the sciatic nerve and DRG after CCI

Since we did not find a reduction of CCI-induced hyperalgesia when therapeutic NOX4 inhibition was started after CCI, we next investigated the kinetics of *Nox4* gene expression in ipsilateral, proximal sciatic nerve segments, L4/L5 DRG, and lumbar spinal cord from WT mice after CCI or sham surgery from Day 1 to 35 by real-time qRT-PCR analysis. We found a significant increase in *Nox4* mRNA after CCI in sciatic nerve on Days 4 (*P* < 0.05) and 8 (*P* < 0.01), which later decreased to baseline levels. In sham-operated mice, *Nox4* mRNA was constitutively expressed at low levels (Fig. 2A). In ipsilateral L4/L5 DRG neurons, we found upregulation of *Nox4* mRNA on Day 4 after CCI to levels twofold higher than in sham-operated controls (*P* < 0.01; Fig. 2B). In lumbar spinal cord, expression of *Nox4* mRNA was unchanged at any time after CCI (Fig. 2C). These data suggest a potential role of NOX4 in the local pathological signaling processes early after nerve damage.

3.3. Mechanical allodynia after CCI is reduced by NOX4-deficiency

Since *Nox4* mRNA expression is increased early after CCI we then re-investigated whether and to which time point NOX4 is involved in the generation of neuropathic pain after CCI by using another NOX4-deficient mouse model (Kleinschnitz et al., 2010). Therefore, *Nox4*^{-/-} mice and *Nox4*^{+/+} littermate controls were tested for pain behavior after CCI. Before CCI, *Nox4*^{-/-} mice and controls had similar baseline levels of mechanical withdrawal thresholds and heat-induced withdrawal thresholds. After CCI, *Nox4*^{-/-} mice had significantly reduced mechanical withdrawal thresholds throughout the entire experimental period beginning from early stages after CCI (*P* < 0.05 for Days 4, 8, 14,

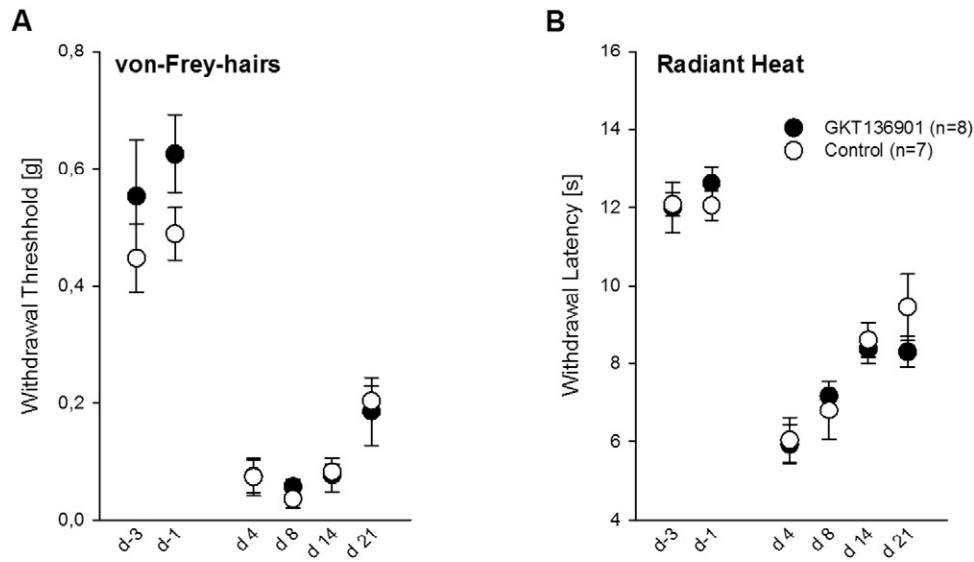


Fig. 1. Neuropathic pain behavior is unchanged after systemic treatment with the dual NOX1/4 inhibitor GKT136901. Manifestation and time course of mechanical (A) and thermal (B) hyperalgesia was similar in C57BL/6 wild-type mice treated with GKT136901 and those treated with vehicle (control). Two-way ANOVA followed by a post-hoc Holm-Sidak pairwise multiple comparison procedure.

21, and 28 after CCI). In *Nox4*^{-/-} mice withdrawal thresholds returned to nearly baseline levels on Day 35 after CCI, whereas *Nox4*^{+/+} mice still showed markedly reduced withdrawal thresholds ($P < 0.01$ for Day 35; Fig. 3A). Withdrawal latencies to radiant heat were not different between the experimental groups (Fig. 3B). Thus, NOX4 seems to be involved in the early development and maintenance of mechanical allodynia following CCI.

3.4. CCI-induced expression of NOX2 and proinflammatory cytokine *Tnf-α* is reduced in *Nox4*^{-/-} mice

To further elucidate the mechanisms of reduced pain behavior in NOX4-deficient animals, we performed a temporal gene expression profile of NOX2 in the sciatic nerve, ipsilateral DRG, and lumbar spinal cord in NOX4-deficient mice and control littermates (*Nox4*^{+/+}) after

CCI and sham surgery. NOX2 is another physiologically relevant NOX isoform that contributes to neuropathic pain hypersensitivity in mice (Kallenborn-Gerhardt et al., 2014; Kim et al., 2010). Further we analyzed prototypic pro- and anti-inflammatory cytokines that are known to be inflammatory markers in the CCI mouse model and that are involved in the induction and maintenance of neuropathic pain (Sommer and Kress, 2004). In *Nox4*^{+/+} mice, we found that CCI-induced upregulation of *Nox2* expression in the sciatic nerve increased to a maximum on Days 4 and 8 after CCI that was approximately 20 times the value on Day 1 and returned to basal levels at later stages, e.g. Day 21 and 37 ($P < 0.01$ for Day 4; $P < 0.001$ for Day 8; Fig. 4). In *Nox4*^{-/-} mice, *Nox2* mRNA levels were only gradually, but not significantly increased in the sciatic nerve after CCI compared to sham-treated animals. Accordingly, direct comparison between *Nox4*^{-/-} and *Nox4*^{+/+} mice of *Nox2* transcription levels after CCI revealed a remarkable

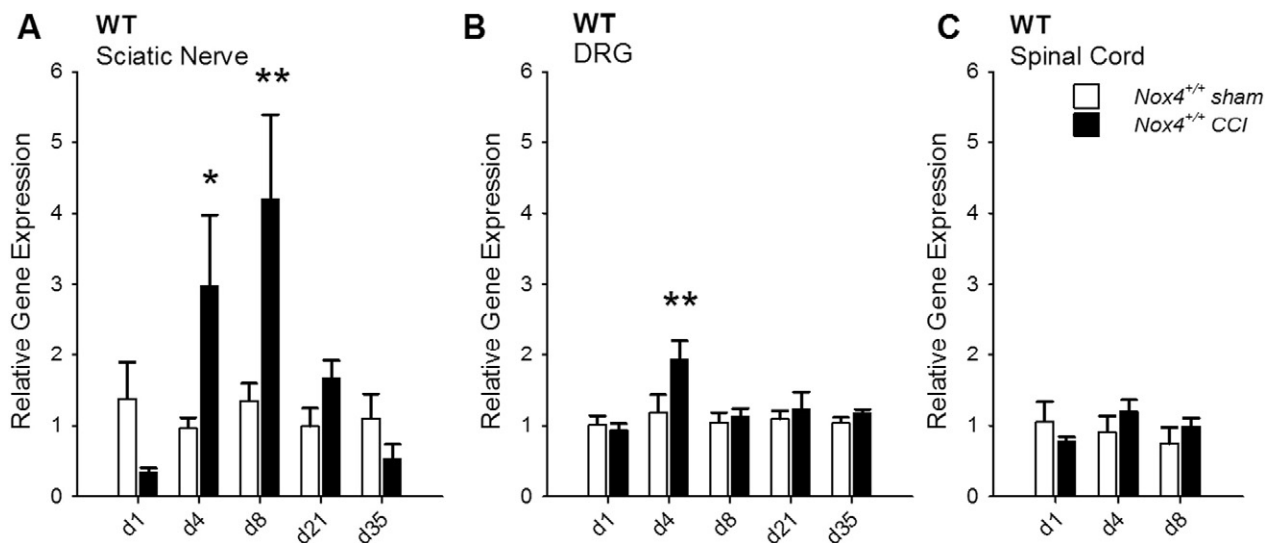


Fig. 2. NOX4 is upregulated early after CCI in mice. Relative gene expression levels of *Nox4* in the ipsilateral, proximal sciatic nerve segments (A), ipsilateral L4/L5 DRG (B), and lumbar spinal cord (C) of wild-type (WT) (*Nox4*^{+/+}) mice on Days 1, 4, 8, 21, and 35 after sham operation or CCI as determined by qRT-PCR analysis. The relative amount of *Nox4* mRNA in the sciatic nerve significantly increased already on Days 4 and 8 and normalized thereafter. In the DRG, *Nox4* mRNA was upregulated after CCI on Day 4 and returned to baseline on Day 8. No significant differences in *Nox4* expression were observed in the lumbar spinal cord after CCI. $N = 4-7$ mice per group and time point. * $P < 0.05$; ** $P < 0.01$; 2-way ANOVA followed by Bonferroni multiple comparison test compared with the control group (sham-operated mice).

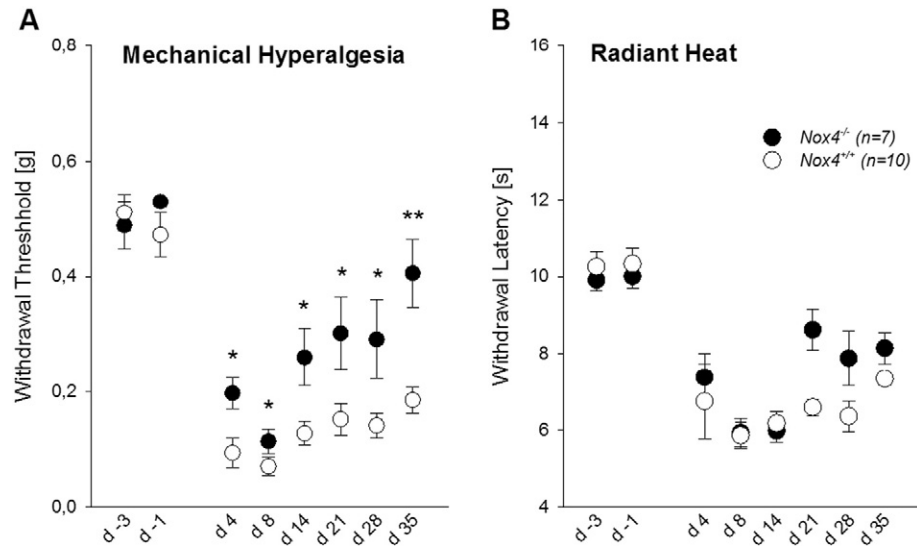


Fig. 3. Neuropathic pain behavior is reduced in NOX4-deficient mice following CCI. (A) Mechanical withdrawal thresholds (von Frey test on the ipsilateral hind paw) of $Nox4^{+/+}$ and $Nox4^{-/-}$ mice are shown before and after CCI. Baseline levels were similar in both groups. After CCI, withdrawal threshold was reduced in both groups. In $Nox4^{-/-}$ mice, the decrease in withdrawal thresholds was significant throughout the observation period. On Day 35 after CCI, the withdrawal threshold in $Nox4^{-/-}$ mice was close to the baseline value, whereas in $Nox4^{+/+}$ mice mechanical hyperalgesia remained prevalent. (B) Thermal hyperalgesia as revealed by sensitivity to radiant heat on a hind paw was unchanged in both experimental groups. * $P < 0.05$; ** $P < 0.01$; 2-way ANOVA followed by a post-hoc Holm-Sidak pairwise multiple comparison procedure.

Nox2 Gene Expression

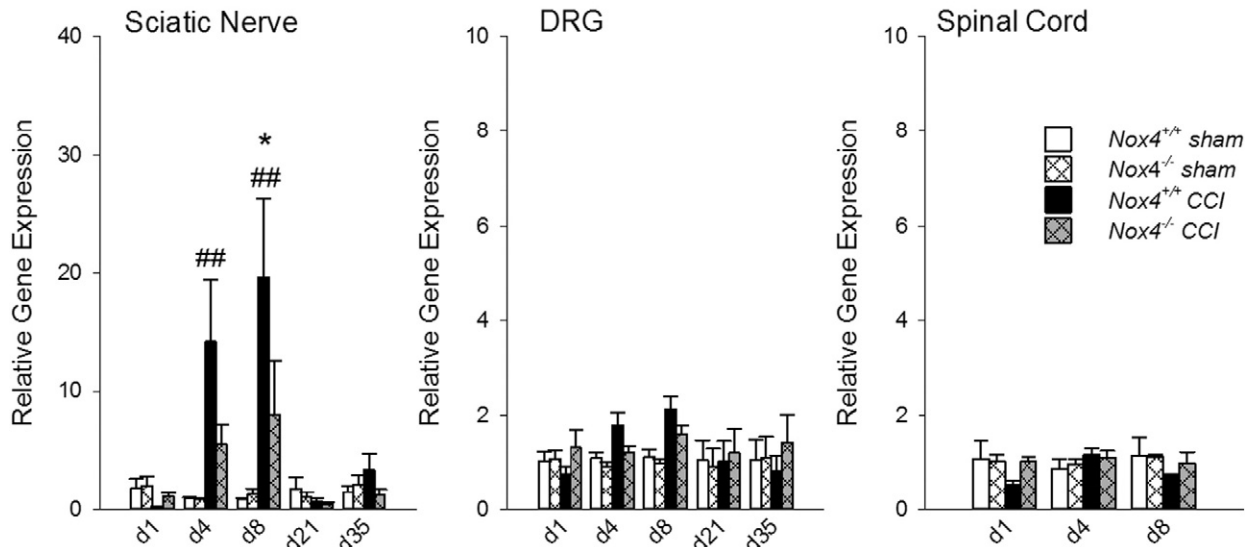
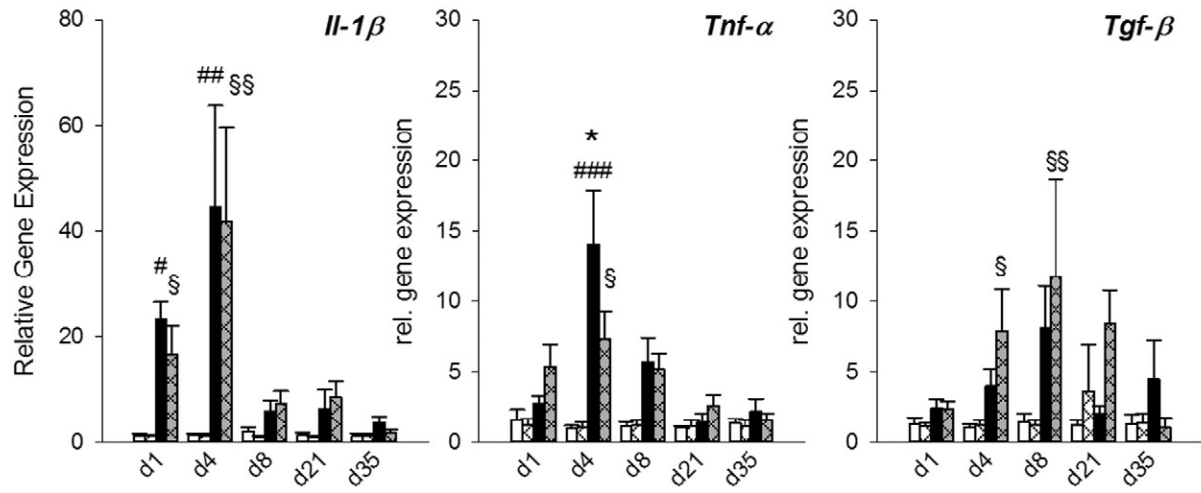
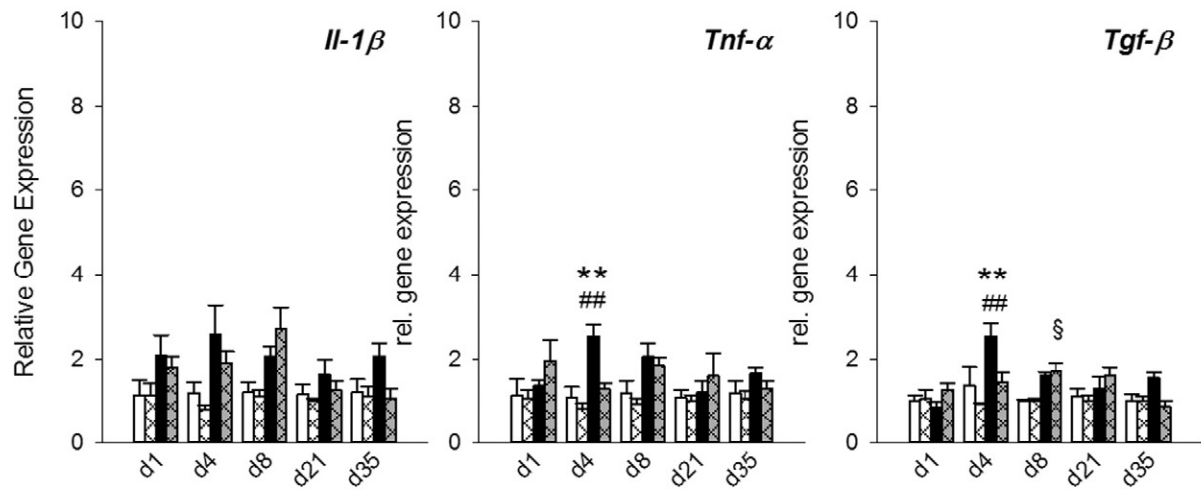
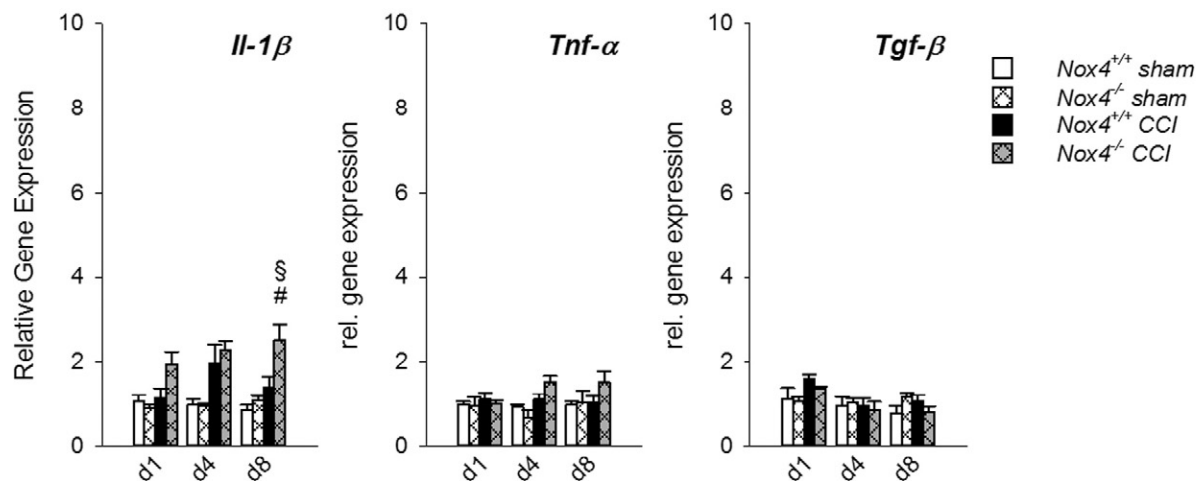


Fig. 4. NOX4-deficiency reduces CCI-induced NOX2 upregulation. Relative gene expression levels of *Nox2* in the ipsilateral, proximal sciatic nerve segments (A), ipsilateral L4/L5 DRG (B), and lumbar spinal cord (C) of $Nox4^{-/-}$ and $Nox4^{+/+}$ mice on Days 1, 4, 8 (Days 21 and 35 not shown) after sham operation or CCI as determined by qRT-PCR analysis. In the sciatic nerve, *Nox2* was strongly upregulated in $Nox4^{+/+}$ mice after CCI to a maximum on Days 4 and 8 of about 20 times the value on Day 1. *Nox2* gene expression also increased in the sciatic nerve of $Nox4^{-/-}$ mice after CCI, but was significantly lower as compared to that of $Nox4^{+/+}$ mice after CCI. In the DRG and spinal cord, *Nox2* mRNA expression was very mild after CCI irrespective of the genotype ($Nox4^{+/+}$ or $Nox4^{-/-}$) or the time after surgery. $N = 4-7$ mice per group and timepoint. * $P < 0.05$ for $Nox4^{+/+}$ vs. $Nox4^{-/-}$ mice after CCI; ## $P < 0.01$ for $Nox4^{+/+}$ mice after CCI vs. sham operation; 2-way ANOVA followed by Bonferroni multiple comparison test compared to the respective control group (sham-operated mice or $Nox4^{+/+}$ mice).

Fig. 5. NOX4-deficiency alleviates CCI-induced early inflammatory reactions in the sciatic nerve and DRG. (A) Relative gene expression levels of *Il-1 β* , *Tnf- α* , and *Tgf- β 1* in the ipsilateral, proximal sciatic nerve segments of $Nox4^{-/-}$ and $Nox4^{+/+}$ mice on Days 1, 4, 8, 21, and 35 after sham operation or CCI as determined by qRT-PCR analysis. In ipsilateral sciatic nerve, CCI led to a robust and early induction of gene expression of proinflammatory *Il-1 β* and *Tnf- α* in $Nox4^{+/+}$ and $Nox4^{-/-}$ mice with a maximum on Day 4, which then decreased from Day 8. The maximal CCI-induced increase in *Tnf- α* on Day 4 was lower in $Nox4^{-/-}$ mice compared to $Nox4^{+/+}$ mice. Gene expression of anti-inflammatory *Tgf- β 1* culminated on Days 4 and 8, and was significantly enhanced in $Nox4^{-/-}$ mice after CCI compared to sham operated mice. (B) Relative gene expression of *Il-1 β* , *Tnf- α* , and *Tgf- β 1* in the ipsilateral DRG L4/L5 of $Nox4^{-/-}$ and $Nox4^{+/+}$ mice. CCI-induced increase of the pro- and anti-inflammatory cytokines in ipsilateral DRG occurred later after CCI and was less pronounced than in the sciatic nerve. Similarly, maximal increase in *Tnf- α* on Day 4 after CCI was significantly lower in $Nox4^{-/-}$ mice compared to $Nox4^{+/+}$ mice. Transcription of *Tgf- β 1* was significantly increased in $Nox4^{+/+}$ mice on Day 4 after CCI compared to $Nox4^{-/-}$ mice. On Day 8 after CCI, *Tgf- β 1* is significantly increased in $Nox4^{-/-}$ mice compared to $Nox4^{+/+}$ mice. (C) Relative gene expression of *Il-1 β* , *Tnf- α* , and *Tgf- β 1* in the lumbar spinal cord. Pro- and anti-inflammatory cytokines were constitutively expressed at low levels without relevant changes in both CCI groups. $N = 4-7$ mice per group and timepoint. * $P < 0.05$, ** $P < 0.01$ for $Nox4^{+/+}$ vs. $Nox4^{-/-}$ mice after CCI; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ for $Nox4^{+/+}$ mice after CCI vs. sham operation; § $P < 0.05$, §§ $P < 0.01$ for $Nox4^{-/-}$ mice after CCI vs. sham operation; 2-way ANOVA followed by Bonferroni multiple comparison test compared with the respective control group (sham-operated mice or $Nox4^{+/+}$ mice).

A Sciatic Nerve**B DRG****C Spinal Cord**

downregulation of *Nox2* in the NOX4-deficient mice on Day 8 ($P < 0.05$). In the DRG or spinal cord we did not observe significant differences between *Nox4*^{-/-} and *Nox4*^{+/+} mice in the expression of *Nox2* mRNA after CCI or sham operation at any time point (Fig. 4).

Further, CCI led to an early robust increase in mRNA expression of the proinflammatory cytokines *Il-1β* and *Tnf-α* in the injured nerve with a maximum on Days 1 and 4 after CCI and decreasing from Day 8, compared to the sham-operated control groups (*Il-1β*: $P < 0.05$ for Day 1 and $P < 0.01$ for Day 4; *Tnf-α*: $P < 0.001$ for Day 4; Fig. 5A). This pattern of upregulation was also present in the DRG but was less pronounced (*Tnf-α*: $P < 0.01$ for Day 4; Fig. 5B). Following CCI, the expression of *Il-1β* in the sciatic nerve of *Nox4*^{-/-} mice was not significantly different to that of *Nox4*^{+/+} mice (Fig. 5A). In contrast, on Day 4 after CCI, the expression of *Tnf-α* in the sciatic nerve and DRG of *Nox4*^{-/-} mice was significantly lower as compared to *Nox4*^{+/+} mice ($P < 0.05$; Fig. 5A,B). Along with the massive increase in proinflammatory cytokines, we also detected an upregulation of the anti-inflammatory cytokine *Tgf-β1* on Days 4 and 8 after CCI in the injured sciatic nerve and DRG (Fig. 5A,B). While *Tgf-β1* transcription levels in the sciatic nerve did not differ significantly between *Nox4*^{+/+} mice subjected to CCI and sham operation, CCI induced a significant upregulation of anti-inflammatory *Tgf-β1* in the injured sciatic nerve of NOX4-deficient mice on Days 4 and 8 ($P < 0.01$ for Day 4 and $P < 0.05$ for Day 8; Fig. 5A). In lumbar spinal cord, we observed only marginal changes in cytokine expression irrespective of the genotype or time after CCI (Fig. 5C). Thus, NOX4 signaling may be important in the regulation of early modulatory inflammatory processes leading to neuropathic pain development following nerve lesion.

3.5. NOX4-deficiency attenuates oxidative and nitrative stress in the lesioned sciatic nerve in the acute phase after CCI

NOX4 is responsible for the formation of ROS, i.e. superoxide and hydrogen peroxide, which can interact with nitric oxide to form RNS. To assess the generation and involvement of oxidative and nitrative stress in early development of neuropathic pain after CCI we stained cross-sections of sciatic nerves to evaluate deposition of DHE and nitrotyrosine, two broad-spectrum indicators of oxidative and nitrative stress (Fig. 6). On Day 1 after CCI, we detected significantly lower amounts of ROS in the sciatic nerve of *Nox4*^{-/-} mice compared to *Nox4*^{+/+} mice as quantified by the mean fluorescence intensity of oxidized DHE ($P < 0.05$; Fig. 6A). Since DHE staining may also be a marker for oxidative chemistry events in general, e.g. formation of peroxynitrite and nitration of the amino acid tyrosine, we further analyzed the extent of protein nitration in *Nox4*^{-/-} and *Nox4*^{+/+} mice on Day 1 after CCI. In accordance with reduced DHE detection in the injured nerve of NOX4-deficient mice, we also found significantly reduced nitrotyrosine-positive cells in these mice compared to *Nox4*^{+/+} mice ($P < 0.05$; Fig. 6B).

4. Discussion

The main finding of the study is that the ROS-producing enzyme NOX4 is important in the early generation of neuropathic pain following peripheral nerve lesions in mice by locally generating oxidative stress products very soon after CCI. Deficiency in NOX4 leads to a reduction in local proinflammatory cytokine upregulation and to an attenuation of mechanical hypersensitivity. Importantly, post-injury pharmacological inhibition of NOX4 after the nerve lesion did not result in any improvement of disease signs, suggesting that NOX4 activation is crucial at the initial stages of neuropathic pain development.

NADPH oxidases were identified as key contributors to oxidative stress and ROS-mediated cell death in pain (Kallenborn-Gerhardt et al., 2012, 2014; Kim et al., 2010) and other neurological disorders such as epilepsy (Williams et al., 2015), brain trauma (Ansari et al., 2014) and stroke (Kleinschmitz et al., 2010). In a spinal nerve transection model NOX2 was responsible for peripheral injury-induced spinal cord

microglia activation, ROS formation and subsequent pain hypersensitivity (Kim et al., 2010). NOX2-deficient mice displayed significantly reduced expression levels of the proinflammatory cytokines *Il-1β* and *Tnf-α*, which is in agreement with our observation of a reduced inflammatory response in NOX4-deficient mice after peripheral nerve injury. Interestingly, in the spinal nerve transection model, *Nox2* gene expression was observed not only in dorsal horn neurons but predominantly in spinal cord microglia dependent on toll-like receptor 2 signaling, and nuclear factor-κB and p38 mitogen-activated protein kinase regulation (Kim et al., 2010; Lim et al., 2013). In contrast, the NOX isoforms 1, 3, and 4 as well as Duox1 and 2, are not expressed in spinal cord microglia following spinal nerve transection (Kim et al., 2010). NOX1 was reported to hold a central function in the development of inflammatory pain by inducing ROS production in DRG neurons leading to enhanced transient receptor potential vanilloid 1 activity and increased sensitivity to painful stimuli (Ibi et al., 2008). In contrast to NOX1, NOX4 was suggested to contribute to the maintenance of neuropathic pain after peripheral nerve injury (Kallenborn-Gerhardt et al., 2014; Kim et al., 2010). In a recent study with a different NOX4 KO mouse model as used here Kallenborn-Gerhardt et al. reported that loss of NOX4 reduced late-phase neuropathic pain and that tamoxifen-induced deletion of *Nox4* induced analgesia in already existing later pain states in the spared nerve injury (SNI) neuropathic pain model (Kallenborn-Gerhardt et al., 2012). Based on these findings we tried to evaluate the therapeutic effect of the pharmacological NOX1/4 inhibitor GKT136901 after CCI (Teixeira et al., 2016; Vendrov et al., 2010). Surprisingly, we did not find any effect of systemic NOX1/4 blockade in behavioral analysis. Therefore, we analyzed again the extent and the kinetics of NOX4 signaling and found upregulation of *Nox4* gene expression and formation of ROS and RNS very early after CCI. Accordingly, neuropathic pain behavior as well as NOX2 expression and ROS and RNS production was significantly reversed in NOX4 KO mice. Our findings of NOX4 upregulation predominantly at the lesion site in the peripheral nerve and much less in the spinal ganglia and spinal cord point toward a primary role of NOX4 in direct development of neuropathic pain instead of modifying secondary processes of central sensitization in higher pain processing structures. Additionally, we identified a reduction of early local proinflammatory cytokine upregulation as an important beneficial effect of NOX4-deficiency in our CCI model. We observed a reduced expression of proinflammatory cytokines *Il-1β* and *Tnf-α*, as well as an increased expression of anti-inflammatory *Tgf-β* in the sciatic nerves and DRG from Day 1 to 8. The reduction of local NOX2 expression and protein nitration in the lesioned nerve was slightly more pronounced than the downregulation of pro-inflammatory cytokines and reduction of neuropathic pain behavior. This is explained as NOX2 signaling and nitrosylation are directly linked to NOX4 deletion which in turn may then influence local inflammation and finally neuropathic pain behavior. It is well established that increased levels of proinflammatory cytokines in the lesioned nerves may change axonal properties, worsen outcome, and thus strengthen chronic pain conditions (Sommer and Kress, 2004). It has been shown in several experimental studies that the acute response in the lesioned nerve is associated with the development of neuropathic pain. Injection of pro-inflammatory cytokines in healthy nerves in equivalent doses to those determined after nerve lesion induce pain behavior (Zelenka et al., 2005) whereas cytokine inhibitors applied locally to the nerve reduce pain behavior (Sommer et al., 2001a, 2001b). Also systemic antagonism of pro-inflammatory cytokines or administration of anti-inflammatory cytokines reduces neuropathic hyperalgesia in animal models (Schäfers and Sommer, 2007). Importantly, these local inflammatory changes are also observed in humans, e.g. in sural nerve biopsies of patients suffering from painful neuropathy (Empl et al., 2001; Uceyler et al., 2007, 2015).

We used prolene sutures in this mouse model, which results in stable neuropathic pain-related behavior and induces local inflammatory changes as reported previously (Ruscheweyh et al., 2007; Schäfers et al., 2002). The original CCI model was described using chromic gut sutures (Bennett and Xie, 1988). In the past, loose ligatures performed

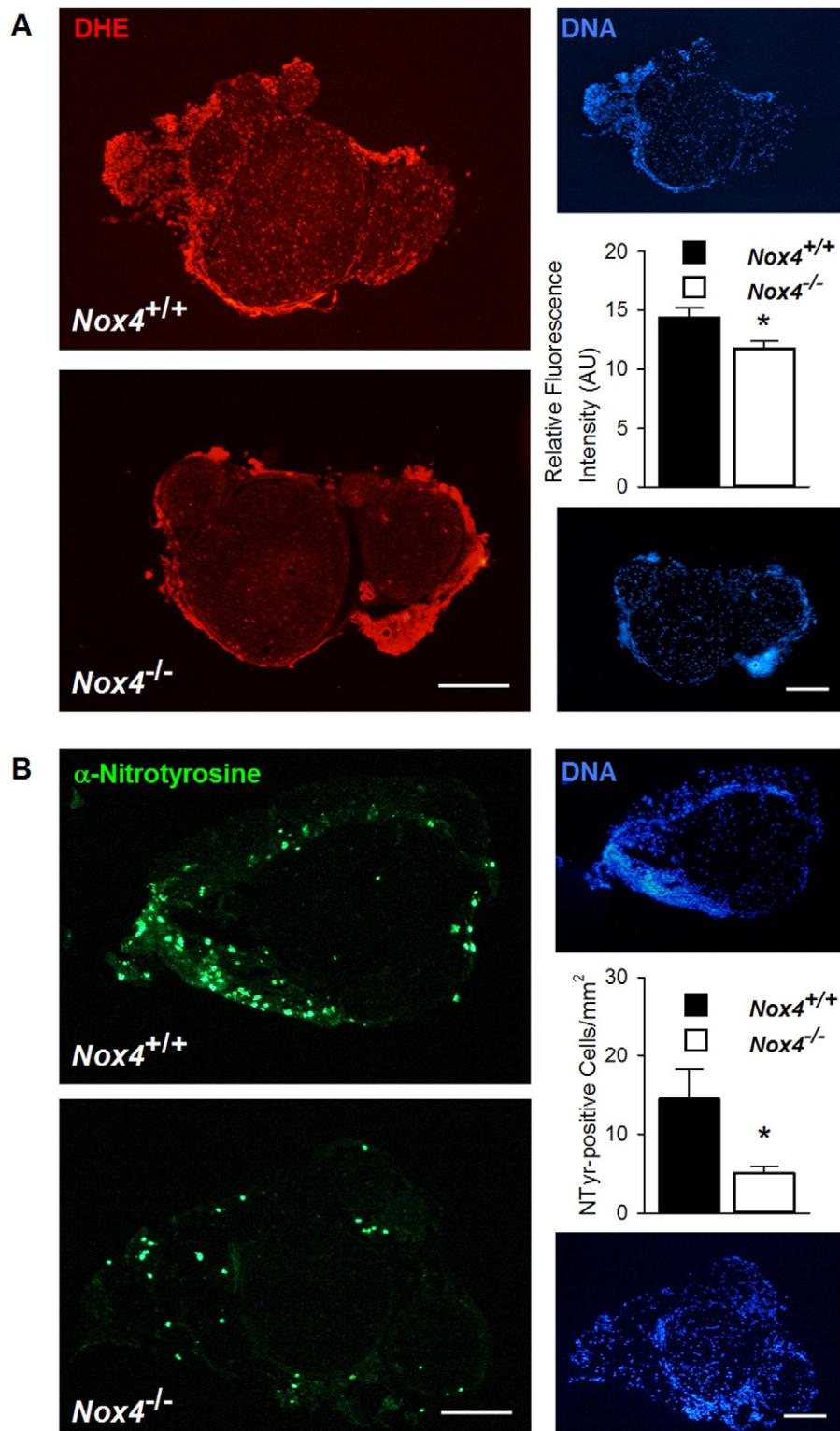


Fig. 6. NOX4-deficiency reduces the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the sciatic nerve in the acute phase after CCI. (A, left) Representative cross-sections of ipsilateral sciatic nerves from *Nox4*^{+/+} and *Nox4*^{-/-} mice on Day 1 after CCI stained for ROS using dihydroethidium (DHE). (A, right middle) Quantification of oxidized ethidium revealed significantly lower ROS in the sciatic nerve of *Nox4*^{-/-} mice on Day 1 after CCI compared to *Nox4*^{+/+} mice. (B, left) Representative cross-sections of ipsilateral sciatic nerves from *Nox4*^{+/+} and *Nox4*^{-/-} mice on Day 1 after CCI stained for RNS using antibodies against α -nitrotyrosine (NTyr). (B, right middle) Quantification of NTyr-positive cells/mm² revealed significantly reduced RNS in the injured nerves and the perineural tissue of *Nox4*^{-/-} mice on Day 1 after CCI compared to *Nox4*^{+/+} mice. Hoechst stain (blue) was used to visualize DNA. *N* = 6–8 mice. **P* < 0.05. Scale bar represents 200 μ m. AU, arbitrary unit.

with simple (non-chromic) gut and with silk, were shown to be less efficient in inducing pain behavior than chromic gut (Maves et al., 1993). This discrepancy is most likely caused by the technique of tying the

ligatures. While we use “loosely constrictive” ligatures that lead to partial Wallerian degeneration of the nerve (Sommer et al., 1993, 1995) no matter which type of ligature is used, others have used very loose

ligatures, not constricting the nerve, or have simply put chromic gut near the nerve (Maves et al., 1993). In the latter case, a chemical component was implied to interact with the nerve and to induce hyperalgesia. In contrast, if the ligatures are tied in a way to induce actual axonal damage, the type of ligature is less important.

Since post-injury inflammation is a key-mediator of neuropathic pain development and maintenance of the painful condition (Kleinschnitz et al., 2006; Sommer and Kress, 2004), our data support the notion that targeting NOX4 may be a promising therapeutic intervention. Indeed, blocking NOX4 function by genetic deletion or pharmacological inhibition proves to be effective also in other disease models, i.e. in models of atherosclerosis (Vendrov et al., 2010), type 2 diabetes (Sedeek et al., 2013), liver inflammation (Lan et al., 2015), ischemic retinopathy (Deliyanti and Wilkinson-Berka, 2015), and of epithelial ciliary dysfunction in neutrophilic asthma (Wan et al., 2016).

Since we describe importance of NOX4 signaling at early stages in the development of neuropathic pain and others found beneficial effects of NOX4 deletion in another mouse model in late stages of neuropathic pain, we now suggest that future studies should aim to define the importance and to evaluate a possible therapeutic window for a pharmacological approach of NOX4 inhibition in patients with nerve lesions and neuropathic pain. This could be achieved by analysis of nerve biopsies or surgery material after nerve trauma at different stages after injury or neuropathic pain development. Thereafter, animal models with matching time courses of NOX4 regulation, ROS production, cytokine regulation, and development of neuropathic pain should be explored for further pharmacological studies.

In principle, oral delivery of GKT136901 at a dose of 10 mg/kg once daily from Day 1 to 21 may also be inadequate for our CCI model. However, the dose and timing of application was adapted from previous studies (Vendrov et al., 2010) that proved to be efficient for systemic NOX4 inhibition and showed beneficial effects in atherosclerotic mice. Another theoretical possibility for the ineffectiveness of GKT136901 is that this dual NOX1/4 inhibitor may generally not be suitable for successful treatment of neuropathic pain given that beside NOX1 and 4 also the NOX isoforms 2 is upregulated after pain induction. However, this is relativized by our observation that CCI-induced *Nox2* upregulation is impaired in our NOX4 KO mice. We also cannot exclude that pain relief in the NOX4 deficient mice is due to reduction in NOX2 signaling. However, as mentioned above and in contrast to our findings of NOX4 upregulation and cytokine dysregulation in the sciatic nerve, upregulation of *Nox2* gene expression is mainly observed in microglia of the spinal cord in neuropathic pain models (Kallenborn-Gerhardt et al., 2014; Kim et al., 2010). Organ- or cell-type specific function is a remarkable prerequisite of the NOX enzymes, especially of NOX4, which is suggested to provoke selective ischemia-reperfusion injury primarily in the brain (Kleinschnitz et al., 2010) and, to a much lesser extent, also in other organs (Braunersreuther et al., 2013; Wilkinson-Berka et al., 2014).

5. Conclusion

Our study describes NOX4 as a major contributor to the initiation of neuropathic pain at early stages after nerve lesion. This is accompanied by a combined induction of inflammatory mediators and oxidative stress mechanisms. Since holistic NOX4 blockade per se is beneficial but post-acute NOX inhibition seems to be ineffective in neuropathic pain development in this mouse model, future studies should focus on evaluating the role and time course of NOX4 signaling in neuropathic pain in humans to identify possibilities for pharmacological intervention.

Conflicts of interest

None.

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